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Supplemental Information

CITED2 Cooperates with ISL1 and Promotes Cardiac Differentiation of Mouse Embryonic Stem Cells

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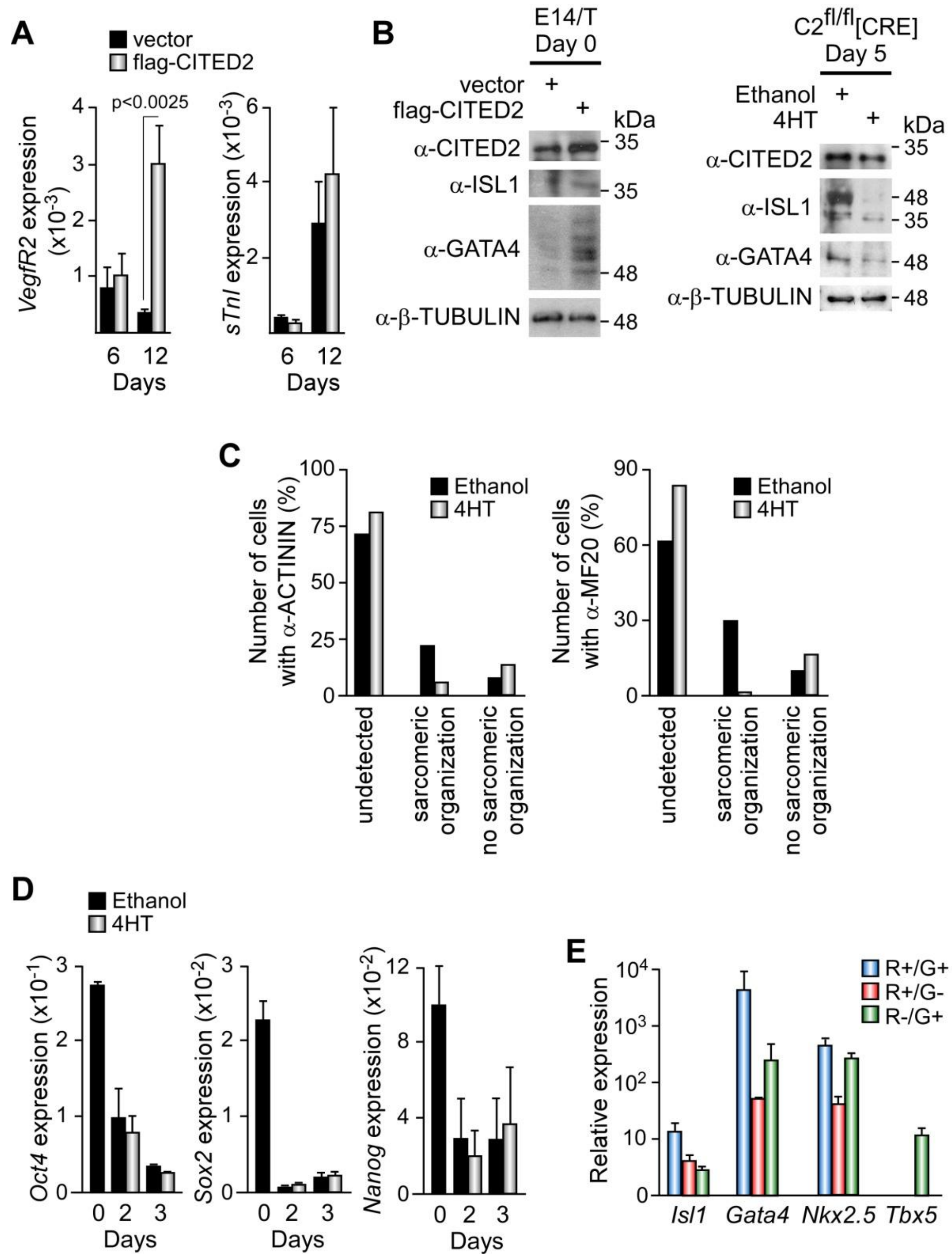
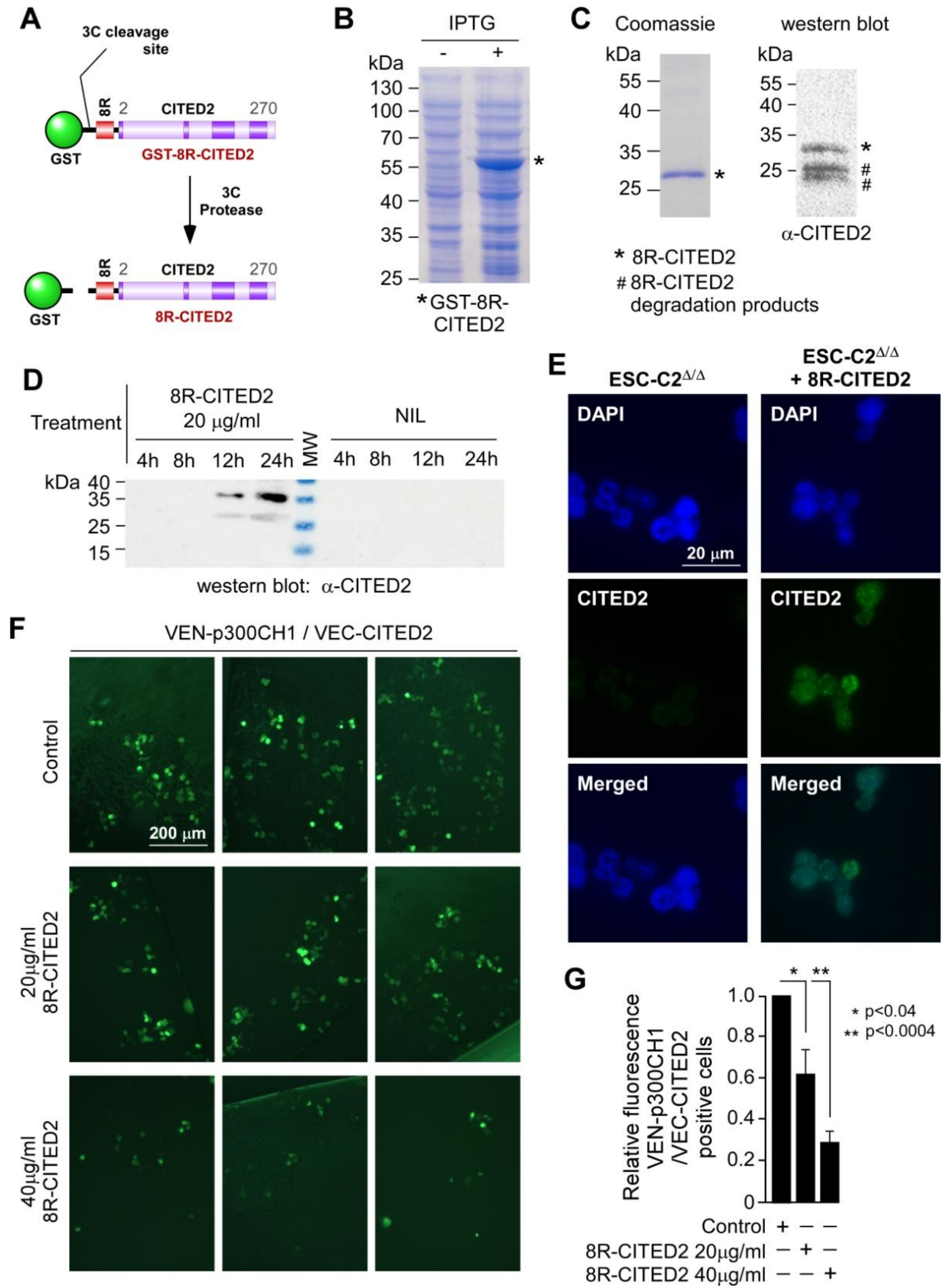
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3

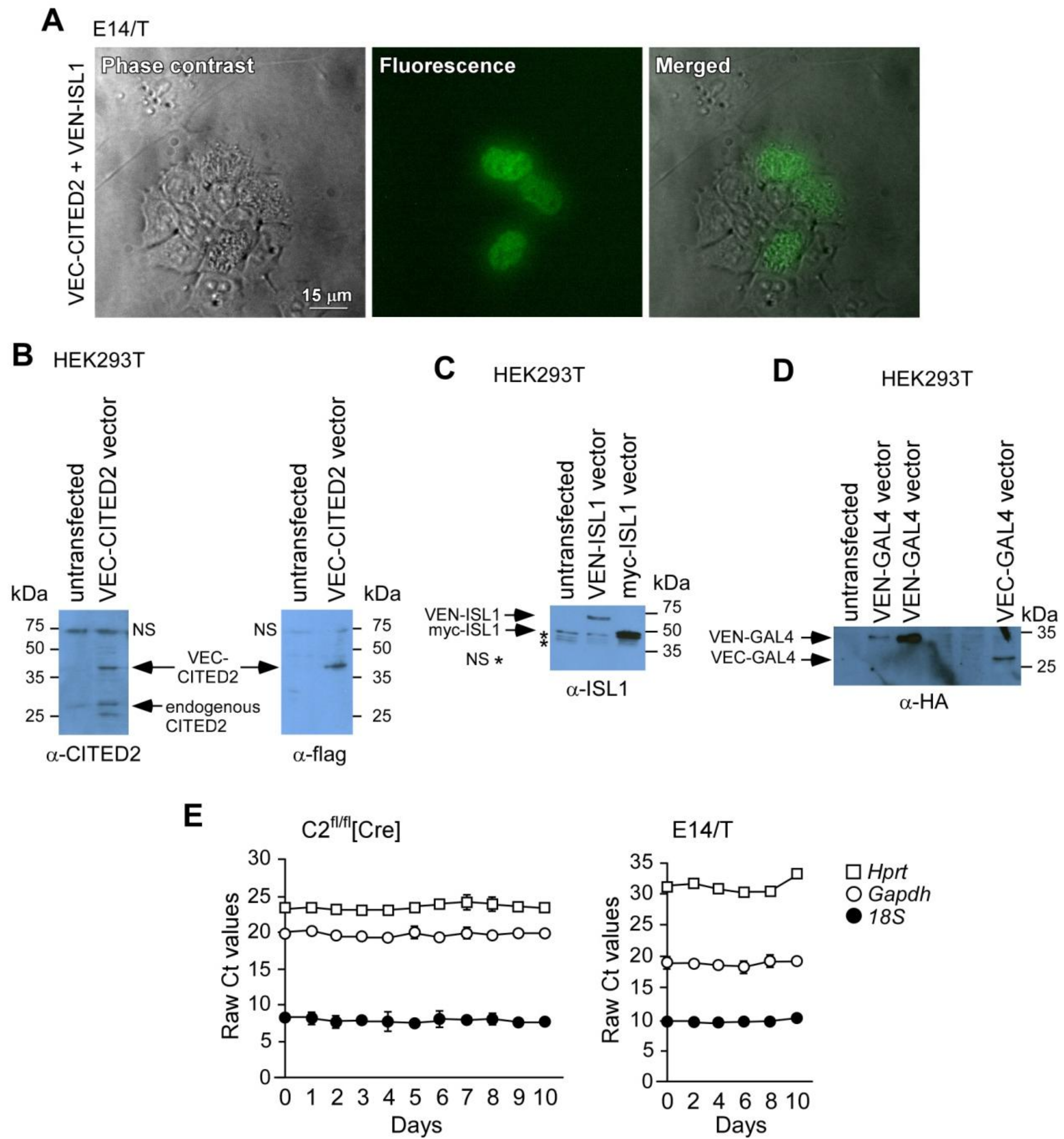


Table S1 – Primers used for qPCR and ChIP qPCR

qPCR	Forward primer	Reverse primer	Reference
18S	CGTCTGCCCTATCAACTTTCG	CCTTGGATGTGGTAGCCGTT	(Chen et al. 2007)
Activin A	CTCCACGATCATGTTCTGAAT	GATGATGTTTTGACCATCATC	(Orimo et al. 1996)
Afp	GCCACCGAGGAGGAAGTG	AGTCTTCTTGCGTGCCAGC	(Gouon-Evans et al. 2006)
Brachyury	CTCTAATGTCCTCCCTTGTTGCC	TGCAGATTGTCTTTGGCTACTTTG	(Ivanova et al. 2006)
Cited2	CTCTAATGTCCTCCCTTGTTGCC	CGCTCGTGGCATTCATGTTG	(Chen et al. 2007)
cTnT	GAGGAGGTGGTGGAGGAGTA	GGCTTCTTCATCAGGACCAA	(Sachinidis and Schwengberg 2006)
Foxa2	GGCCCAGTCACGAACAAAGC	CCCAAAGTCTCCACTCAGCCTC	(Ivanova et al. 2006)
Gapdh	TCCCACTCTTCCACCTTCGATGC	GGGTCTGGGATGGAAATTGTGAGG	(Ivanova et al. 2006)
Gata4	TTCCTGCTCGGACTTGGGAC	TTCCCAGGCAGGTGGAGAATAAG	(Ivanova et al. 2006)
Isl1	CTTAAGCATGCCCTGTAGCTGG	CAGACAGGAGTCAAACACAATCCC	(Ivanova et al. 2006)
Hprt	GTTGGATACAGGCCAGACTTTGTTG	GAGGGTAGGCTGGCCTATAGGCT	(Chen et al. 2007)
Mesp1	TGTACGCAGAAACAGCATCC	TTGTCCCCTCCACTCTTCAG	(Zhong and Jin 2009)
Myh6	GATGGCACAGAAGATGCTGA	CTGCCCCTTGGTGACATACT	(Sachinidis and Schwengberg S 2006)
Nanog	CTCATCAATGCCTGCAGTTTTTCA	CTCCTCAGGGCCCTTGTCAGC	(Zhong and Jin 2009)
Nestin	GGTGCTGAGTATGTCGTGGA	CGGAGATGATGACCCTTTTG	(Sachinidis and Schwengbreg 2006)
Nkx2.5	CCACTCTCTGCTACCCACCT	CCAGGTTCAGGATGTCTTTGA	(Sachinidis and Schwengberg S 2006)
Nodal	TGGCGTACATGTTGAGCCTCT	TGAAAGTCCAGTTCTGTCCGG	(Ogawa et al. 2007)
Oct4	GCAGGAGCACGAGTGGAAAGCAAC	CCAGGCCTCGAAGCGACAGATG	(Ivanova et al. 2006)
Sox2	CGAGATAAACATGGCAATCAAATG	AACGTTTGCCTTAAACAAGACCAC	(Ivanova et al. 2006)
sTnI	GGAAATCCAAGATCACTGCCTCC	GGGCACTGAGGGACAGACCA	(Martínez-Fernandez et al. 2006)
Tbx5	GGACCCAGTCCCTTGAATGG	TCCAGGCTGAGGAGTTCTAGGC	(Ivanova et al. 2006)
VegfR2	ACTGCAGTGATTGCCATGTTCT	TCATTGGCCCGCTTAACG	(Fujimori et al. 2008)
βIII-tubulin	G TATTCAGGCCCGACAACTTT	GGGTGTCAACCAGAGGAAGT	(Suter et al. 2009)

ChIP qPCR	Forward primer	Reverse primer	Reference
Gata4	ACTCCCTTAGGCCAGTCAGC	GGAAAAGAGCAGGGACTCG	(Snyder et al. 2010)
Gapdh	CAAGGCTGTGGGCAAGGT	TCACCACCTTCTTGATGTCATCA	(Chen et al. 2012)
Isl1 A	TTTTGGGTCTAACCGTCCTACTC	CCGCTTTCCTTCACTGACTC	(Nimura et al. 2009)
Isl1 B	ACTATTTGCCACCTAGCCACAG	AGAGGGAGTAATGTCCACAGTG	(Nimura et al. 2009)
Nkx2.5	AGGCAAAGAAATCACTCCACA	TGTTACAATGGCTGGGAAGG	(Snyder et al. 2010)
Tbx5	GAAGCATTTTCTATACTTTGTGAGA	TCAGCCAGCTGTTTTCAGAG	(Snyder et al. 2010)

Supplemental Figure Legends

Figure S1. Related to Figures 1, 2 and 3. The modulation of the expression of CITED2 at the onset of differentiation affects the expression of *VegfR2*, ISL1 and GATA4, α–ACTININ and MYOSIN HEAVY CHAIN (MF20), but does not impair the expression of *sTnI* and of genes involved in the maintenance of pluripotency. (A) *VegfR2* and *sTnI* expression detected by qPCR from extracts isolated from E14/T at D6 and D12 of differentiation in cells expressing normal levels of CITED2 (vector) or overexpressing flag-CITED2. (B) Detection of CITED2, ISL1 and GATA4 protein levels by western blotting in extracts from E14/T ESC transiently transfected with a control- or a flag-CITED2 expression vector (left), and in in extracts from C2fl/fl[Cre] cells differentiated for 5 days and treated with Ethanol or 4HT at D0 for 2 days (right). Loading in each lane was monitored by detection of β-TUBULIN. (C) Percentage of cells either unstained, stained for sarcomeric α–ACTININ (left) or MYOSIN HEAVY CHAIN (right - MF20) and presenting some sarcomeric organization, or presenting a poor expression of α–ACTININ or MF20 and no sarcomeric organization in cells described in Figure 2G. (D) Expression of pluripotency markers (*Oct4*, *Sox2* and *Nanog*) determined by qPCR at D0, D2 and D3 of differentiation in cultures derived from $C2^{fl/fl}$[Cre] ESC treated with 1μM 4HT or ethanol at D0 for 48 hrs, and normalized for *Gapdh*. (E) Relative expression of *Isl1*, *Gata4*, *Nkx2.5* and *Tbx5* determined by qPCR and normalized for *Gapdh* in cell populations derived from AD2 at D6 differentiation as described in Figure 4B. Expression of the indicated genes is reported relative to their expression in non-cardiac progenitor cells R-/G- set at 1. Note the logarithmic axis scale. Results in panels A, D, E are presented as the mean ± s.e.m. of three independent biological experiments, while results in panel C are presented as the mean ± s.e.m. of two independent biological experiments.

Figure S2. Related to Figure 3. Production and characterization of 8R-CITED2 recombinant protein. (A) Schematic representation of the chimeric GST-8R-CITED2 protein produced in bacteria. Oligonucleotides with a sequence corresponding to the 8 arginines (8R) cDNA and a fragment encoding the human CITED2 were cloned in the pGEX6P1 vector (GE Healthcare Life Sciences) to express a chimeric protein consisting of the Glutathione S-transferase (GST) linked to 8R, which are themselves fused to CITED2 (GST-8R-CITED2). The 3C cleavage site encoded at the C-terminal part of the GST by the pGEX6P1 vector is indicated. (B) Detection by SDS-PAGE separation and Coomassie staining of total proteins from BL21 *E.coli* transformed with the GST-8R-CITED2 expression vector and either stimulated (+) or unstimulated (-) by IPTG. The position of GST-8R-CITED2 protein is indicated by an asterisk (*). (C) Detection by SDS-PAGE separation and either Coomassie staining (left panel) or western blotting (right panel) of 8R-CITED2 purified recombinant protein after purification by affinity column and cleavage on column by the Rhonivirus 3C protease coupled to GST. The recombinant 8R-CITED2 (*) and degradation products (#) are indicated. The western blotting was performed with the anti-CITED2 antibody as described in the main text. Cells not supplemented with 8R-CITED2 (NIL) were loaded as control (D) Transduction of 8R-CITED2 into $C2^{\Delta/\Delta}$[MG5] *Cited2*-null ESC described elsewhere (Kranc et al., 2015). Purified 8R-CITED2 was added in the culture medium at a final concentration of 20μg/ml. Detection by western blotting of intracellular 8R-

CITED2 in whole cellular extracts prepared at the indicated times after addition of 8R-CITED2 or in control cells using the anti-CITED2 antibody as described in the main text. (E) Accumulation of 8R-CITED2 in the cellular nuclei of C2$^{\Delta/\Delta}$[MG5] *Cited2*-null ESC detected by immunohistochemical reaction against CITED2 24 hrs after supplementation of 8R-CITED2 as described in D. (F) Fluorescence detection in HEK293T cells co-transfected with plasmids expressing VEN-p300CH1 and VEC-CITED2 (25 ng each), and supplemented in the culture medium 24 hrs post-transfection with 8R-CITED2 at the final concentration of 0 (Control), 20 and 40μg/ml. The morphology of transfected cells (not shown) and the fluorescence signal obtained by BiFC (green panels) were visualized 48 hrs after transfection. (G) Quantification of BiFC detected in HEK293T cells treated as indicated in F. The number of fluorescent cells is presented relative to the fluorescence detected in the control condition (VEN-p300CH1/VEC-CITED2) set to 1. Results are presented as the mean ± s.e.m. of three independent biological experiments.

Figure S3. Related to Figure 5. CITED2 and ISL1 interaction visualized in living cells by BiFC assays and test expression of fusion proteins. (A) Undifferentiated E14/T ESC transfected with VEN-ISL1 and VEC-CITED2 expression vectors examined one day after transfection for morphological aspect (Phase contrast), and fluorescence emission (Fluorescence). The merged picture is also presented. (B-D) Whole cell extracts from untransfectedHEK293T cells or transiently expressing the indicated proteins were analysed by western blotting. (B) Expression of VEC-CITED2 detected with anti-CITED2 (left panel) and anti-flag (right panel) antibodies in HEK293T tranfected cells. The position of VEC-CITED2 fusion protein (~40kDa), endogenous CITED2 (~30 kDa) and non-specific protein (NS) are indicated. (C) Expression of VEN-ISL1 (~56 kDa) and myc-ISL1 (~40 kDa) detected with anti-ISL1 antibody. The positions of fusion proteins and non-specific proteins (*, NS) are indicated. (D) Expression of VEN-GAL4 (~34 kDa) and VEC-GAL4 (~27 kDa) analysed with anti-HA antibody. The positions of the fusion proteins are indicated. (E) The reference genes *Hprt*, *Gapdh* and *18S* were assayed across the cDNA samples prepared from undifferentiated (D0) C2$^{fl/fl}$[Cre] (left) and E14/T ESC, or differentiated cells up to the time points indicated. The mean of raw threshold cycle (Ct) values obtained from three biological replicates are plotted.

Table S1. Related to all figures. – Primers used for qPCR and ChIP qPCR

Supplemental Experimental Procedures

Embryonic stem cells, culture conditions and isolation of cardiac progenitor populations

Apple D2 (AD2), $C2^{fl/fl}$, $C2^{\Delta/\Delta}$[LA11], $C2^{fl/fl}$[Cre] and E14/T mouse ESC lines were described previously, and were cultured on gelatine-coated plates in undifferentiating medium supplemented with LIF. $C2^{fl/fl}$ ESC harbour both *Cited2* alleles functional and $C2^{\Delta/\Delta}$[LA11] ESC are *Cited2*-knockout cells originated from $C2^{fl/fl}$ ESC. $C2^{fl/fl}$[Cre] ESC which have the exon2 of *Cited2* flanked by LoxP sites and constitutively express a tamoxifen-inducible Cre recombinase were treated with 1μM of 4-hydroxytamoxifen (4HT) during 48 hrs at the indicated time points to delete *Cited2* gene or with Ethanol used as a 4HT vehicle control. All ESC lines were differentiated using the hanging-drop method in medium containing 20% FBS without LIF supplementation (differentiation medium). Briefly, 500 (for E14/T or AD2) or 1000 (for $C2^{fl/fl}$, $C2^{\Delta/\Delta}$[LA11] or $C2^{fl/fl}$[Cre]) cells were cultured in 20μl hanging drops of differentiation medium for 48 hrs to initiate EB formation. Next, EB were grown in differentiation medium in suspension for 3 days in a bacterial petri dish before transfer to 0.1% gelatine coated plates. The puromycin added to the culture medium of undifferentiated E14/T cells to sustain the presence of high levels of flag-CITED2 expressing plasmid (pPyCAGIP-flagCITED2) or the control vector (pPyCAGIP) in these cells as previously described (Kranc et al., 2015), was omitted in the differentiation medium. Progression of differentiation was monitored with inverted microscopes. R+G+, R-G+, R+G- and R-G- labelled cell populations were isolated by FACS from E9.5 *Nkx2.5-eGFP*/SHF-*dsRed* double transgenic heart embryos or AD2-derived cells as previously described (Domian et al., 2009). All mice were cared for within the Animal Care facilities of the Massachusetts General Hospital under the supervision of an active and functioning Subcommittee on Research Animal Care (SRAC), which serves as the Institutional Animal Care and Use Committee

(IACUC) as required by the Public Health Service (PHS) Policy on Humane Welfare Regulations.

Immunochemistry

Immunocytochemistry was performed with $C2^{fl/fl}$[Cre] ESC treated with ethanol or 4HT at D0 and differentiated for 10 days. At D10, cells were dissociated by trypsinization and grown for 24 hours on coverslips coated with 0.1% GELATIN, before being washed with phosphate buffered saline (PBS; Sigma), fixed in 4% formaldehyde (Sigma) for 15 minutes, permeabilized in 0.1% Triton X-100 diluted in PBS (Sigma) for 20 minutes, and blocked at room temperature with a 2% BOVINE SERUM ALBUMIN (BSA; Nzytech) in PBS for at 30 minutes. Samples were then incubated either with an anti-α-ACTININ (sarcomeric) antibody (A7811, Sigma; at 1:500 dilution) or with an anti-sarcomeric MYOSIN (DSHB, MF20; at 1:300 dilution) monoclonal primary antibodies diluted in blocking solution for 2 hours at room temperature. The coverslips were then washed three times in PBS and incubated for 1 hour at room temperature with the AlexaFluor-594 donkey anti-mouse antibodies (Invitrogen) used at 1:500 dilution in blocking solution. The coverslips were then washed three times with PBS and placed onto slides using mounting medium containing DAPI (Mowiol-DAPI). Fluorescence microscopy was performed using an Axio Imager Z2 Fluorescence microscope (Carl Zeiss) at a 100x magnification. Negative controls were used to set up exposure conditions for detection of a specific signal. Western blotting assays were performed using 20μg of whole cell lysates prepared from the indicated mouse ESC as previously described (Kranc et al., 2015). Mouse monoclonal JA22 against CITED2 (AB5155, Abcam), anti-ISL1 (AB109517, Abcam), and anti-flagM2 (F1804, Sigma) mouse monoclonal antibodies were used at 1:2000 dilution. Mouse monoclonal anti-GATA4 (sc-25310, Santa Cruz) was used at 1:200 dilution. Loading was monitored by probing

the membrane with a mouse monoclonal anti-β-TUBULIN antibody (T5293, Sigma) used at 1:5000 dilution.

Chromatin immunoprecipitation (ChIP) assays

$1x10^8$ of E14/T cells transfected with a plasmid expressing flag-CITED2 (pPyCAGIP-flagCITED2) or control vector (pPyCAGIP), or untransfected E14/T ESC-derived cells at D5 of differentiation dissociated to a single cells by 0.05% TRYPSIN (SIGMA) at 37°C for 15 min, were fixed with 1% formaldehyde and quenched by glycine to final concentration of 0.125 M (SIGMA). Nuclei were extracted and submitted to 75U of Microccocal Nuclease-MNase (Fermentas) for DNA fragmentation. CITED2 and flag-CITED2 immunoprecipitations were performed with rabbit polyclonal anti-CITED2 (H-220, Santa Cruz Biotechnology) and monoclonal anti-flagM2 (F1804, Sigma) antibodies, respectively. A rabbit IgG-ChIP grade (AB46540, Abcam) was used for control immunoprecipitations of endogenous CITED2. The co-immunoprecipitated DNA was purified by phenol:chloroform:isoamyl extraction and precipitation. ChIP experiments to determine the presence of H3triMek4 were performed using mouse monoclonal anti-H3triMek4 (AB10812, Abcam) and anti-flagM2 (F1804, Sigma) as previously described (Kranc et al., 2015). The enrichment of target genomic elements was determined by qPCR as previously described (Kranc et al., 2015), using primers listed in Supplemental Table S1.

Production and transduction of the recombinant 8R-CITED2 protein

Full-length human CITED2 cDNA and an oligonucleotide encoding 8 arginines (8R) were cloned into the pGEX6P1 vector (GE Healthcare Life Sciences) to express a chimeric protein consisting of the Glutathione S-transferase (GST) in fusion with the 8R domain and CITED2 (termed GST-8R-CITED2, Figure S2). The construct harbours

also a 3C cleavage site encoded at the C-terminal part of the GST. GST-8R-CITED2 protein was expressed in BL21 *E. coli* cells, purified by Gluthatione Sepharose Fast Flow (GE Healthcare) resin affinity chromatography using a fast protein liquid chromatography (FPLC) ÄKTA™ (Amersham Biosciences). GST-8R-CITED2 was cleaved on the affinity column by the Rhonivirus 3C protease coupled to GST for 16 hrs at 4ºC. Subsequently, the recombinant 8R-CITED2 was eluted from the column and stored at -80ºC. For cellular transduction, the protein solutions were thawed and diluted with the culture medium 24 hrs and stored at 4ºC prior to supplementation of cell cultures at final concentration of 5-10μg/ml. Newly constructed plasmids were validated by sequencing and the expression of fusion proteins tested by western blot (Figure S2). Details of plasmid construction and protein purification are available upon request.

Protein interactions and plasmids

For *in vitro* binding assays, GST-CITED2 fusion proteins and *in vitro* translated myc-ISL1 were prepared as previously described (Machado-Oliveira et al., 2015). Myc-ISL1 expression plasmid was constructed in pcDNA3 (Invitrogen) with an amino-terminal myc epitope tag. Plasmids expressing flag-CITED2 and pPyCAGIP were previously described (Chen et al., 2012). For BiFC assays, plasmids expressing VEC-CITED2, VEN-p300CH1, VEC-GAL4, VEN-GAL4 and the VEN-vector were described elsewhere (Machado-Oliveira et al., 2015). The plasmid VEN-ISL1 expressing VEN in fusion with ISL1 was obtained by subcloning ISL1 cDNA fragment of the Myc-ISL1 expression plasmid into the VEN-vector in frame with the VEN domain. All newly constructed plasmids were validated by sequencing and the expression of fusion proteins tested by western blot (Figure S3). Details of plasmid construction are available upon request. For co-immunoprecipitation assays, ~0.4mg of whole cell extracts from HEK293T cells transfected with flag-CITED2 expression vector alone or together with myc-ISL1 vector, were prepared in a buffer containing 50 mM Tris pH7.5, 100 mM NaCl, 15 mM EGTA,

0.1% Triton-X100, and Complete protease inhibitors (Roche), and incubated overnight at 4°C with monoclonal anti-flagM2 covalently coupled to agarose beads (Sigma). Immunoprecipitates were washed five times, and eluted by competition with flag peptide (Sigma) added at 200 μg/ml for 30 minutes at 4ºC. Eluted samples were subjected to western blot analysis. For BiFC, $2x10^5$ E14/T ESC were transfected with 250 ng of the indicated vectors and examined for fluorescence emission two days after transfection in undifferentiated cells, or at D2 and D5 of differentiation by EB formation.

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